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Whole cigarette smoke promotes human gingival epithelial cell apoptosis and inhibits cell repair processes

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Background and Objective: Smoking cigarettes increases the risk of developing various types of human diseases, including cancers and periodontitis. As gingival epithelial cells are known to play an active role in innate immunity via the secretion of a wide variety of mediators, and as these cells are the first ones exposed to environmental stimuli such as cigarette smoke, we sought to investigate the effects of whole cigarette smoke on normal human gingival epithelial cells and tissue.

Material and Methods: Human gingival epithelial cells were extracted from healthy nonsmokers and used either as a monolayer or as an engineered human oral mucosa to investigate the effect of whole cigarette smoke on cell growth, apoptosis and wound repair/migration.

Results: Our findings show that when gingival epithelial cells were exposed once to whole cigarette smoke, this resulted in a significant inhibition of cell growth through an apoptotic pathway, as confirmed by an increase of Bax and a decrease of Bcl-xL and caspase-3 activity. Cigarette smoke also inhibited epithelial cell migration. These effects may explain the disorganization of the engineered human oral mucosa tissue when exposed to whole cigarette smoke.

Conclusion: Exposure to whole cigarette smoke markedly inhibits epithelial cell growth through an apoptosis/necrosis pathway that involves Bax and Bcl-xL proteins and caspase-3 activity. Cigarette smoke also disrupts epithelial cell migration, which may negatively affect periodontal wound healing.

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Cigarette smoking constitutes a well-established risk factor for multiple diseases, including periodontitis (1,2). Smokers exhibit an increased prevalence and severity of periodontal disease (3,4). These are documented by a significant incidence of tooth mortality/loss (5), early onset of disease (4,6) and increased rates of disease progression (7). Furthermore, clinical investigations have demonstrated that cigarette smoking may hamper the healing outcome following surgical and nonsurgical periodontal therapy (8,9). Indeed, smokers have been shown to demonstrate a considerably less favourable response compared with nonsmokers following scaling and root planning or modified Widman flap surgery, as well as during periodontal maintenance following active therapy. Compromised healing following regenerative therapy in intrabony and gingival-recession defects in

smokers has also been reported (10,11). These adverse effects on human tissue and cells are all related to the multiple toxic agents present in cigarette smoke.

Cigarette smoke contains approximately 4800 chemicals, with over 60 of them known to have adverse effects on human cells and tissues. Chemicals found in cigarette smoke are also highly genotoxic and lead to various forms of DNA damage, regardless of the biological system involved (12). Cigarette smoke promotes p53 mutations, resulting in the DNA methylation of several genes (13), including retinoic acid receptor β, H-cadherin, adenomatous polyposis coli, p16INK4a and RAS association domain family 1A (*RASSF1A*) tumour suppressor genes (14). Cigarette smoke also increases telomerase activity (15) and promotes lung cell apoptosis (16).

Most cells targeted by tobacco smoke are bronchial (17) and oral epithelial cells (18). In human bronchial epithelial cells, cigarette smoke condensate induces changes in cell structure and function through alterations in the cell signaling pathways (19). In addition to genetic insults, cigarette smoke constituents also activate biochemical pathways that are associated with apoptosis, cell cycle progression and cell growth (20).

Apoptosis is regulated through different genes, including inducers (*Bax*, *bcl-xS*, *Bad*, *Bak* and *Bik*) and inhibitors of apoptosis (*bcl-2*, *bcl-xL*, *BAG-1* and *mcl-1*) (21,22). Defects in apoptosis-regulating genes may cause human cancers (23). Apoptosis, or programmed cell death, is a tightly regulated process consisting of complex biochemical cascades that involve the activation of caspase-3 through either extrinsic or intrinsic apoptotic pathways (24). Cigarette smoke may inhibit cell proliferation through these apoptotic pathways, as demonstrated with airway epithelial cells (25,26); therefore, inhibiting epithelial and mesenchymal cell proliferation and deregulating programmed cell death processes may hamper cell migration and wound healing.

In the oral cavity, the epithelium forms the first line of defence against toxic agents and bacteria such as periodontopathogens (27). In addition to its function as a protective physicochemical barrier to the outside environment, oral epithelium has a number of metabolic and immunological roles, including fluid and ion transport regulation, mucus production/elimination and participation in innate and adaptive immunity, as well as the modulation of inflammation, cell migration and repair processes (28). These functions are essential to maintaining mucosal homeostasis. Despite the fact that human oral epithelial cells are the first cell type to be exposed to cigarette smoke, few studies have addressed the effect of cigarette smoke on these cells and the possible initiation and development of oral diseases that include cancer and periodontitis. The purpose of this study was thus to investigate the toxic effect of whole cigarette smoke on gingival epithelial cells and cell death pathways and to determine the impact of cigarette smoke on cell migration/wound healing and mucosal structure using normal human gingival epithelial cell monolayer cultures and an engineered human oral mucosa (29).

Material and methods

Materials

1R3F cigarettes were purchased from the Kentucky Tobacco Research and Development Center (Orlando, FL, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Sigma (St Louis, MO, USA), and an Annexin V Fluos staining kit was obtained from Roche (Mannhein, Germany), while the Bax, Bcl-xL and β-actin antibodies were purchased, respectively, from BD systems (Mississauga, ON, Canada), Chemicon (Temecula, CA, USA) and β-actin from (Sigma-Aldrich, ON, Canada). Collagenase-P was obtained from Boehringer Mannheim (Laval, Québec, QC, Canada) and collagen type I was purchased from Gibco-Invitrogen (Burlington, ON, Canada). Finally, normal human gingival epithelial cells and fibroblasts were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA).

Culture of gingival epithelial cells and fibroblasts

Normal human gingival epithelial cells were cultured in Dulbecco's modified Eagle's–Ham's F12 (3:1; DMEH) medium supplemented with 5 μg/mL of human transferrin, 2×10^{-9} M of 3,3′,5′-triiodo-L-thyronine, 0.4 μg/mL of hydrocortisone, 10 ng/mL of epidermal growth factor, 100 IU/mL of penicillin G and 10% fetal bovine serum. The medium was changed three times a week. When the culture reached 90% confluence, the cells were detached from the flasks using a 0.05% trypsin–0.1% ethylenediaminetetraacetic acid (EDTA) solution, were washed twice and resuspended in DMEH-supplemented medium at a final concentration of 10^6 cells/mL. The cells were subsequently used to determine cigarette smoke toxicity. Human gingival fibroblasts were then grown in DMEH medium supplemented with 10% fetal calf serum, 100 IU/mL of penicillin G, 25 μg/mL of streptomycin and 0.5 μg/mL of amphotericin B. Upon 90% confluence, the oral fibroblasts were used between passages two and four to engineer the human oral mucosa.

Engineered human oral mucosa tissue

An engineered human oral mucosa model was created, as previously described (29). Briefly, gingival fibroblasts and epithelial cells were cultured to enable the formation of a complex three-dimensional spatial cellular organization similar to that of normal human oral mucosa. The lamina propria was produced by mixing rat tail collagen type I with gingival fibroblasts, followed by subsequent culture in fetal calf serum-supplemented culture medium for 4 d. The lamina propria was then seeded with gingival epithelial cells to obtain the engineered human oral mucosa. The tissue specimens were grown in submerged conditions until the entire surface of the lamina propria was covered with epithelial cells. To produce stratified epithelium, the engineered human oral mucosa was raised to an air–liquid

interface for an additional 5 days to facilitate the organization of the epithelium into its different strata.

Effect of cigarette smoke on gingival epithelial cell growth

The gingival epithelial cells were detached from 75 cm^2 culture flasks by means of a 0.05% trypsin–0.1% EDTA solution, were washed twice in culture medium and counted, and then seeded onto 35 mm diameter × 10 mm high Petri dishes at 3×10^5 cells per plate in DMEH medium. Following incubation for 24 h in a humidified atmosphere of air containing 5% CO_2 at 37°C, the epithelial cell cultures were placed into a custom-made smoke chamber (Fig. 1). As is shown, a cigarette was placed into one end of a tube linked to the chamber. On the other end, a second tube linked the chamber to a standard vacuum. This allowed the cigarette smoke to penetrate inside the chamber. The quantity/volume of smoke in the chamber was controlled by a valve (Fig. 1). Cultures were placed into the chamber in sterile conditions, and the chamber was hermetically covered prior to burning the cigarette. Cultures were exposed or not to the smoke of one whole cigarette for 5, 15 or 30 min, after which time the cells were fed fresh medium and were cultured for 2 or 4 d, with the medium changed every 24 h. Following these culture periods, epithelial cell viability and growth were assessed by means of a trypan blue exclusion test, as we previously reported (30). Results are reported as the means ± SD of six separate experiments.

Effect of cigarette smoke on gingival epithelial cell apoptosis/necrosis

Normal human gingival epithelial cells were cultured to 80% confluence and exposed to the smoke of one whole cigarette for 15 or 30 min, after which time the medium was refreshed and the cells were cultured for 6 h at 37°C in a humidified atmosphere of air containing 5% CO_2. The cells were then detached from the culture plate by means of a 0.05% trypsin–0.01 EDTA solution. Apoptotic and necrotic cells were determined by annexin V–fluorescein isothiocyanate (FITC) binding assay according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA). Annexin V–FITC and propidium iodide were added to each cell suspension, and the mixtures were incubated for 30 min in the dark at room temperature. The cells were then washed twice with phosphate-buffered saline, and each pellet was suspended in incubation buffer (Roche Diagnostics). Immediately following the staining procedure, sample acquisition was performed using a FACSort cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), with between 30,000 and 50,000 events collected for each sample. Annexin V fluorescence emission was detected in the FL-1 channel and propidium iodide in the FL-2 channel. For each experiment, control analyses were achieved with the following samples: (i) an unstained sample to determine the level of autofluorescence; (ii) an annexin V-labelled cell population with optimal compensation for FITC fluorescence; and (iii) a propidium iodide-labelled population on these settings, which was used to subtract the overlapping propidium iodide fluorescence. The cell population that was annexin V positive, propidium iodide negative was determined as an early apoptotic population, while the cell population that was annexin V positive, propidium iodide positive represented a late-stage apoptotic/necrotic population. The experiment was repeated five separate times.

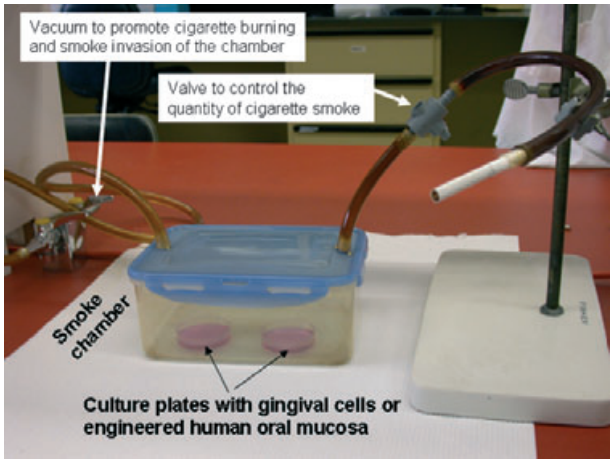

Fig. 1. Photograph of our custom-made cigarette smoke chamber.

Effect of cigarette smoke on Bax and Bcl-xL expression by gingival epithelial cells

Gingival epithelial cells were cultured up to 80% confluence, then exposed to the smoke of one whole cigarette for 15 or 30 min, after which time the culture medium was refreshed and the cells were incubated for 24 h in a humidified atmosphere of air containing 5% CO_2 at 37°C. The cultures were then used to prepare protein cell lysates. To do so, the cells were washed with ice-cold phosphate-buffered saline and lysed using lysis buffer [50 mM of HEPES, pH 7.4; 1% (v/v) Triton X-100; 4 mM of EDTA; 1 mM of sodium fluoride; 0.1 mM of sodium orthovanadate; 1 mM of tetrasodium pyrophosphate; 2 mM of phenylmethylsulfonyl fluoride; 10 μg/mL of leupeptin; and 10 μg/mL of aprotinin]. The lysates were incubated for 60 min on ice and then vortexed, with the insoluble materials removed by centrifugation (14,000*g*, 2 min, 4°C). The protein lysates were subsequently used for immunoblotting, as previously reported (30).

Whole-cell lysates (10 μg of total protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto polyvinyl difluoride membranes (pore size 0.2 μm). The membranes were blocked with 5% bovine serum albumin in Tween-20–Tris-buffered saline (TTBS; Tris-buffered saline, 0.1%

Tween-20) for 1 h at ambient temperature and incubated overnight at 4°C with primary anti-Bax and anti-Bcl-xL antibodies (1:1000 dilution) in TTBS containing 0.5% bovine serum albumin. The membranes were washed three times with TTBS for 10 min, after which they were incubated with horseradish peroxidase-labelled secondary antibody (1:1000 dilution in TTBS) for 1 h at ambient temperature. For protein detection, the membranes were washed for 3 h with Tris-buffered saline, subsequently incubated in Enhanced chemiluminescence (Amersham, Piscataway, NJ, USA), and assessed by means of a FujiFilm Image Reader LAS-1000 Pro (FujiFilm USA, Valhalla, NY, USA). The experiment was repeated five separate times.

Effect of cigarette smoke on caspase-3 activity

Gingival epithelial cells cultured to 80% confluence were exposed to the smoke of one whole cigarette for 15 or 30 min and incubated in a humid atmosphere for an additional 6 h at 37°C. The cells were then washed twice with warm phosphate-buffered saline and treated with lysis buffer containing 10 mM of dithiothreitol for total protein extraction. Following protein quantification, caspase-3 activity was evaluated by means of a Caspase-3/CPP32 Fluorometric Assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions; this assay detects the cleavage of DEVD–AFC (7-amino-4trifluoromethyl coumarin) substrate. Cell lysates (50 μg) were incubated with an equal volume of 50 μM of final concentration DEVD–AFC for 90 min. The samples were then read with a fluorometer (400 nm excitation, 505 nm emission) and were expressed as AFC fluorescence units. The experiment was repeated five separate times.

Monolayer wound repair assay

Wound repair assays were performed as previously described (31). Briefly, gingival epithelial cells were grown to confluence in six-well plates in DMEH medium. Upon confluence, wounds were made in the confluent monolayer of each well using a 200 μL pipette tip. The cultures were then exposed or not to the smoke of one whole cigarette during 15 or 30 min. Following exposure, 2 mL of fresh medium were added to the cultures, which were then incubated for 3, 6, 12 or 24 h in a humidified atmosphere of air containing 5% CO_2 at 37°C. Digital photographs were taken (Coolpix 950; Nikon, Canada, Montreal, QC, Canada), and the percentage of wound closure (epithelial cell migration) was calculated by comparing relative wound areas before and after cigarette smoke exposure using the following formula: (initial scratch size) – (size after an identified culture period) ÷ (initial size) × 100. The experiment was repeated five separate times.

Structural analysis of engineered human oral mucosa following exposure to the smoke of one whole cigarette

In humans, the oral mucosa tissue is in direct contact with smoke components. To mimic this situation, we designed engineered human oral mucosa to investigate the effect of cigarette smoke on tissue structure. The engineered tissues were prepared and exposed or not to the smoke of one whole cigarette for 15 or 30 min, after which time the culture medium was refreshed and the engineered human oral mucosa was incubated for 6 or 24 h. Biopsies were harvested from each engineered tissue, which were then fixed in 4% paraformaldehyde and embedded in paraffin. Thin microtome sections (5 μm thick) were prepared from each biopsy, and haematoxylin and eosin staining was used to evaluate the structure of the different tissues.

Statistical analysis

Experimental values are given as means ± SD. The statistical significance between the control values (nonexposed to cigarette smoke) and the test values (exposed to cigarette smoke) was determined using one-way ANOVA. *Post hoc* comparisons were performed using Tukey's method. Normality and variance assumptions were verified using the Shapiro–Wilk test and the Brown–Forsythe test, respectively. All of the assumptions were fulfilled. *p*-Values were declared significant at 0.05. Data were analysed by means of the SAS version 8.2 statistical package (SAS Institute, Inc., Cary, NC, USA).

Results

Cigarette smoke downregulated epithelial cell proliferation

As shown in Fig. 2, following MTT staining, the absorbance values obtained with the gingival epithelial cells exposed to cigarette smoke showed significantly inhibited cell growth. Indeed, exposure to the smoke for 5, 15 or 30 min, followed by culture for 2 d, showed a reduction in the number of viable cells in all of the whole cigarette smoke-treated cultures. As shown in Fig. 2, the number of viable cells dropped from 3×10^5 (initial seeding concentration) to 3×10^4 cells after 5 min, to almost 1.5×10^4 cells after 15 min, and finally to 10^4 cells after 30 min of exposure to whole cigarette smoke and culture for 2 d. This effect was confirmed at 4 d postexposure to the smoke (Fig. 2). It is important to note that although the whole cigarette smoke was toxic, there remained a significant number of viable cells, which were useful for subsequent analyses.

Cigarette smoke promoted gingival epithelial cell apoptosis

As shown in Fig. 3, the cigarette smoke induced epithelial cell apoptosis after 15 or 30 min of exposure. Figure 3A illustrates the dot plots of annexin V–FITC/propidium iodide, revealing three separate clusters: viable cells (lower left quadrant), early apoptotic cells (lower right quadrant) and necrotic or late apoptotic cells (upper right quadrant). The smoke caused a significant increase in the percentage of necrotic/apoptotic cells (from 3.7% before exposure to 64.3% after 15 min of exposure and 73.9% after 30 min of exposure). This effect was confirmed by

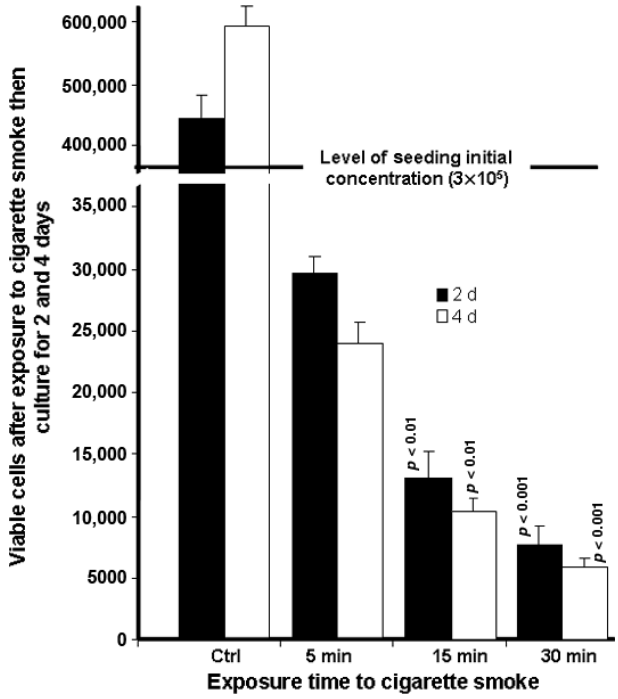

Fig. 2. Gingival epithelial cell toxicity following exposure to whole cigarette smoke. Cells were cultured for 24 h to allow for adhesion and the initiation of proliferation. The cultures were then exposed to the smoke of one whole cigarette for various periods, after which time the cells were incubated in a humidified atmosphere of air containing 5% CO_2 at 37°C for 2 (filled columns) or 4 d (open columns). Following each time point, the cells were subjected to a trypan blue exclusion test. The statistical difference was determined by comparing the cell viability values obtained after exposure for 5 min to those obtained after 15 and 30 min exposure to whole cigarette smoke. Values are means + SD of six separate experiments. The difference was considered significant at $p < 0.05$.

the apoptotic/nonapoptotic protein expression. As shown in Fig. 3B, Bax, an apoptotic protein absent in normal cells, was increased following exposure to the cigarette smoke, whereas Bcl-xL, a nonapoptotic protein expressed by normal cells, was completely inhibited following exposure of the cells to the cigarette smoke.

One explanation for the presence of apoptotic death following stress is the activation of one or more crucial steps (such as caspase activity) along the apoptosis pathway caused by the cigarette smoke components. In order to test this hypothesis, we exposed gingival epithelial cells to cigarette smoke for various periods to determine caspase-3 activity. As shown in Fig. 4, following exposure to cigarette smoke, caspase-3 activity was increased, reaching 10 units after 15 min of exposure and 15 units after 30 min of exposure, compared with the control, in which the activity was approximately 6 units. Overall data demonstrate the negative effect of cigarette smoke on gingival epithelial cells via an apoptotic pathway, and as this smoke reduced cell proliferation through an apoptotic mechanism, this may ultimately disturb cell migration/wound healing.

Cigarette smoke decreased epithelial cell migration/wound healing

Epithelial cells were exposed to the smoke of one cigarette immediately after injury and were assessed at various time points. As shown in Fig. 5A, the exposed epithelial cells migrated progressively over 24 h to cover the edges of the scratch in the form of a confluent monolayer. Although significantly hindered by the presence of the cigarette smoke, this progressive migration occurred at the same rate along the entire length of the wound, as ascertained by optical microscope observations. The scratch even increased in width in the presence of the smoke. Furthermore, it is worthy of mention that cell shape in the smoke-exposed cultures differed from that observed in the unexposed ones. In the smoke-free cultures, the cells were small and cuboidal in shape, whereas in the smoke-exposed cultures, the cells were larger in size and exhibited a faint nucleus (differentiating shape). It is thus important to point out that cigarette smoke inhibited cell migration and increased the wound size. As shown in Fig. 5B, the percentage of healing was lower in the cells exposed to the smoke for 30 min than in those exposed for 15 min. Also, the inhibition of wound healing was greater with longer culture periods (12 and 24 h) than with the shorter early culture period (6 h). The cigarette smoke therefore had a negative effect on both cell migration and wound size.

Cigarette smoke disorganized tissue structure

As shown in Fig. 6, we were able to generate gingival mucosa tissue *in vitro*. The epithelium from the nonstimulated tissue revealed a well-organized structure, with stratified multilayered cells. Following 15 or 30 min of exposure to cigarette smoke and subsequent culture for 6 h, we observed an increase in epithelial disorganization, as determined by cell detachment and a reduced number of epithelial cell layers. The effects were greater with 30 than with 15 min of exposure. Following exposure to the smoke and 24 h of culture, the gingival epithelium was greatly reduced. This confirms the toxic effect of cigarette smoke on gingival cells and tissues.

Discussion

In the present study, the exposure (once) of normal human gingival epithelial cells to whole cigarette smoke resulted in a time-dependent loss of cell growth, as detected by trypan blue exclusion assay. The effect of cigarette smoke on human gingival epithelial

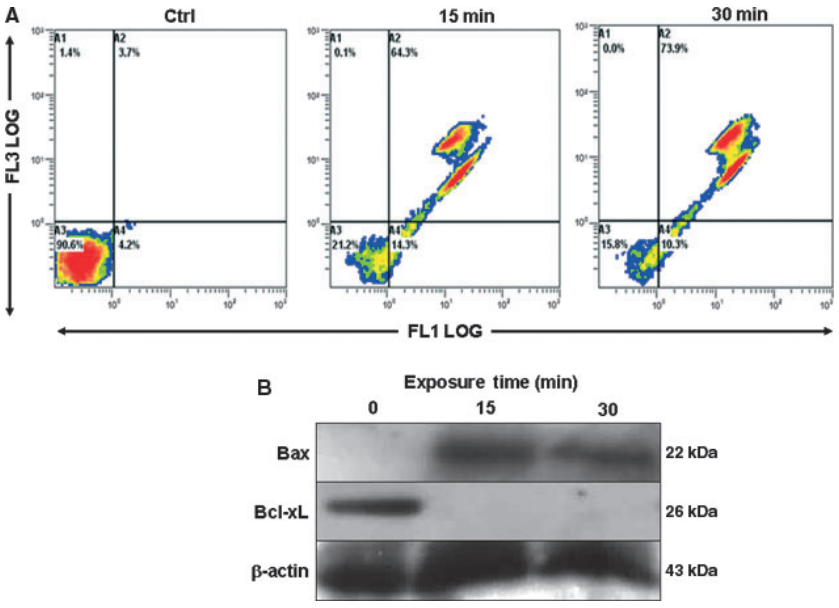

Fig. 3. Effect of cigarette smoke on epithelial cell apoptosis. Flow cytometry analysis of gingival epithelial cell apoptosis following exposure to the smoke of one whole cigarette for various times, and staining with annexin-V/PI. (A) Selected plots from annexin-V/PI-stained cells after exposure to cigarette smoke and subsequent culture for 24 h ($n = 5$). (B) Western blot staining of pro-apoptotic (Bax) and anti-apoptotic proteins (Bcl-xL) following exposure or not to cigarette smoke ($n = 5$). FL, Forward Light Scatter; FL1, for once color and FL/ for the second color; LOG; logarithmic scale.

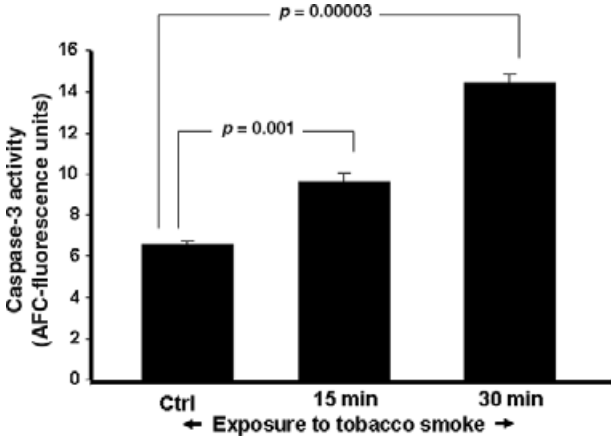

Fig. 4. Gingival epithelial cell caspase-3 activity following exposure to cigarette smoke. Gingival epithelial cells were cultured until 80% confluence, exposed to the smoke of one whole cigarette for various periods, and cultured for an additional 6 h. The cells were then lysed and the cell lysates assayed for caspase-3 activity, as described in the Material and methods. Values are the means + SEM of four experiments ($n = 5$). AFC, 7-Amino-4tri-Fluorom-ethyl Coumarin.

cells may occur through apoptotic and necrotic phenomena. Indeed, the smoke-exposed epithelial cell culture showed a significant apoptotic/necrotic cell density, as ascertained by annexin V/propidium iodide staining. Cigarette smoke therefore had a significant effect on the gingival epithelial cells by reducing the number of viable cells. These results support those previously reported in other models, including human bronchial (32) and nasal epithelial cells (33). Apoptosis is crucial to maintaining normal tissue homeostasis and is in accordance with proliferation and differentiation. Increasing evidence shows that disturbing the balance between apoptosis and proliferation in lung tissue contributes to pulmonary disease (34).

It was reported that in emphysema patients, an increase in apoptotic cells was accompanied by an increased expression of Bax proteins (35). As previously suggested (36), an increased expression of Bax may counter the anti-apoptotic activity of Bcl-2. Our study demonstrates that whole cigarette smoke modulated gingival epithelial cell apoptosis/necrosis through Bax increase and Bcl-xL inhibition. We suggest that the Bax upregulation and Bcl-xL downregulation were involved in the modulation of gingival epithelial cell apoptosis through the disruption of the pro-apoptotic–anti-apoptotic balance in the mitochondrial apoptosis pathway (34). Furthermore, via an opening of permeability transition pores, the activation and translocation of Bax to the mitochondria induced a release of apoptotis-inducing proteins into the cytosol, which ultimately executed the apoptosis (35).

Apoptosis onset is associated with the proteolytic activation of caspases, which play a critical role in triggering apoptosis (36). Caspases are synthesized as proenzymes that are processed by self-proteolysis and/or cleavage by another protease to their active forms in cells undergoing apoptosis (37). Caspase-3, a major trigger of apoptosis, is promoted during the early apoptotic stage, and the activated form is a marker for cells undergoing apoptosis (38). In this study, the gingival epithelial cells exhibited significant caspase-3 activity during the long exposure time to cigarette smoke. Here, caspase-3 smoke-induced activation in the epithelial tissue demonstrated the occurrence of apoptosis and the involvement of caspase-3 in cigarette smoke-induced apoptosis.

In promoting gingival epithelial cell apoptosis/necrosis, cigarette smoke may hamper periodontal wound healing. Nonlethal levels of whole smoke reportedly inhibited fibroblast migration *in vitro*, which is vital to an efficient periodontal healing process (39).

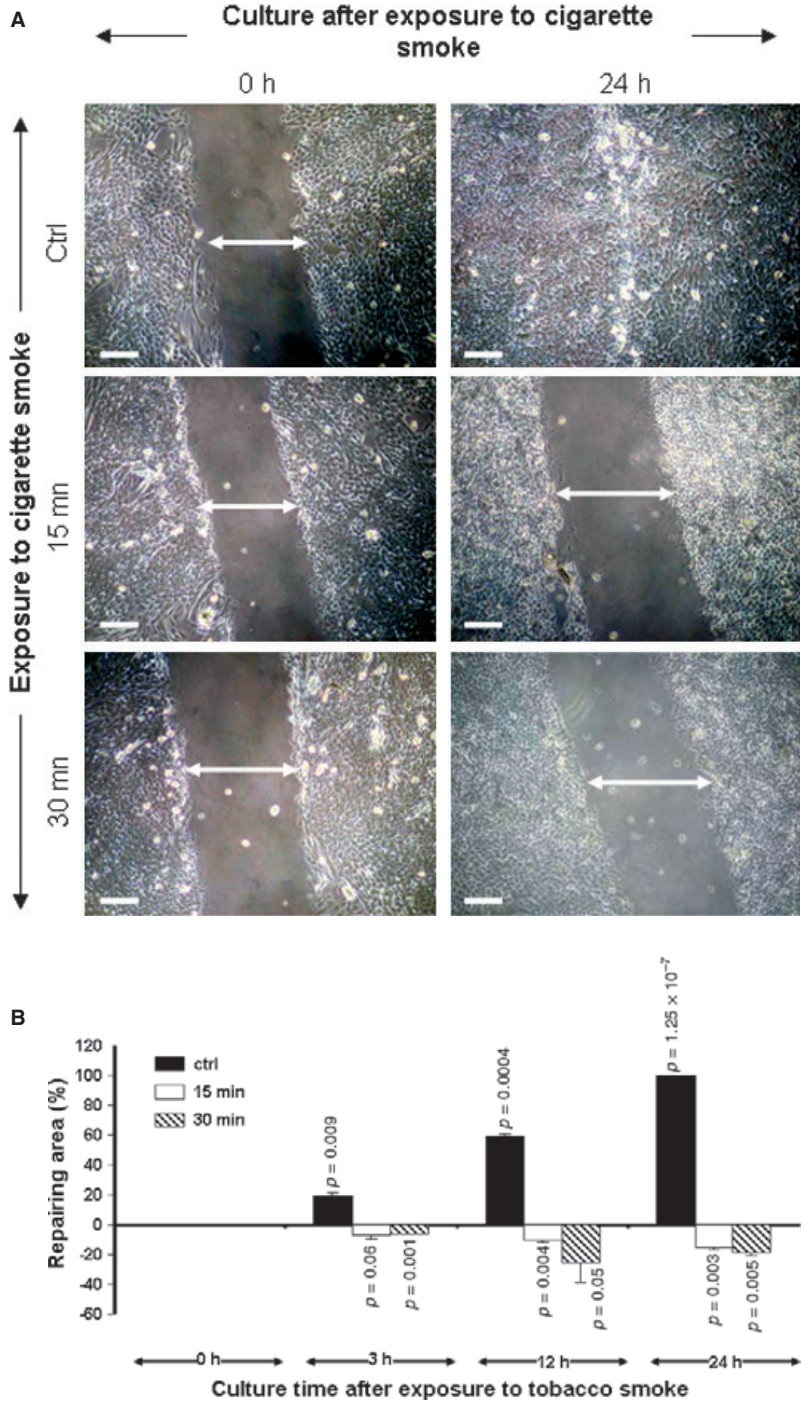

Fig. 5. Effect of cigarette smoke on oral epithelial cell repair. Gingival epithelial cells were cultured to 100% confluence. Scratches were then made on each monolayer and the cultures exposed to the smoke from one whole cigarette for various time points. Following exposure, the medium was refreshed by adding an additional 2 mL, and the cultures were maintained in the appropriate conditions for various times prior to observations and photographs. (A) Scale bars, 50 μm. Arrows indicate the initial and final size of the scratches before and after exposure the smoke or not (Ctrl). (B) The defect recovery of the gingival epithelial cells following exposure to smoke. This was obtained using the following formula: (initial scratch size) – (size after an identified culture period) ÷ (initial size) × 100. Values are means ± SD of five separate experiments ($n = 5$). The difference was considered significant at $p < 0.05$ in comparing the unexposed and cigarette smoke-exposed cultures. In the control, unexposed cell cultures, the scratch wounds healed at the same rate along the entire length of the wound.

The negative effect of cigarette smoke on wound healing may also occur through epithelial cell deregulation. Our study is the first to demonstrate that whole cigarette smoke inhibited gingival epithelial cell migration following a single exposure. As demonstrated with fibroblasts (39), the impact of smoke on gingival epithelial cells may occur through a deregulation of the ATP production pathway, which would thus promote the production and activation of stress response proteins (40). A sustained stimulation of these proteins may promote such diseases as periodontitis and oral cancers (41). Further research will undoubtedly elucidate this mechanism. Exposure to cigarette smoke may also damage oral mucosa structure. As oral mucosa consists of stratified epithelium that constitutes the first layer in contact with cigarette smoke, it was deemed necessary to investigate the effect of this smoke on tissue structure. Using our engineered human oral mucosa model, we showed that exposure to smoke disturbed the epithelial structure, resulting in significant tissue desquamation, particularly with longer exposure periods. An alteration of the epithelium may thus occur through an inhibited production of structural proteins such as E-cadherin. Indeed, cigarette smoke condensate was shown to increase the permeability of respiratory epithelium and to lead to a deregulation of both E-cadherin and tight junctions in animal models (42). A destabilization of E-cadherin expression by cigarette smoke was also demonstrated in oral mucosa cells (43,44). E-cadherin downregulation has been associated with tissue dysfunction similar to that in cancer and periodontal diseases (45). A significant reduction in E-cadherin levels was reported in periodontal disease compared with healthy conditions (46). The decrease in E-cadherin may be due to tissue invasion by periodontopathogens. Future studies using normal human gingival cells and engineered human oral mucosa will no doubt enable us to learn more regarding the cellular and molecular pathways involved in interaction between whole cigarette smoke and human tissues and

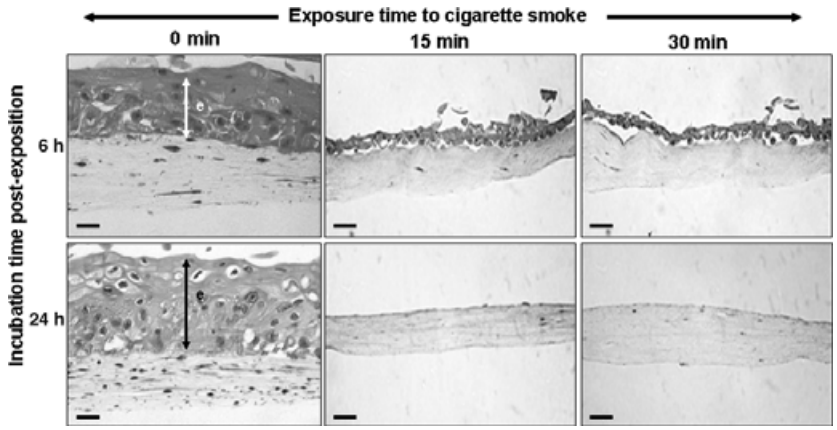

Fig. 6. Effect of the smoke of one whole cigarette on tissue structure. Histological features of the engineered human oral mucosa following exposure to the smoke of one whole cigarette. Engineered tissue was exposed to cigarette smoke for various time periods and subsequently cultured for 6 or 24 h. The collected biopsies were subjected to hematoxylin and eosin staining. Note the absence of epithelium in the samples collected 24 h after exposure to smoke. Representative photographs of four separate experiments are shown (n = 5; two engineered human oral mucosae per experiment). Scale bars, 30 μm. e, epithelium.

disease development. In summary, we demonstrated that one exposure to whole cigarette smoke significantly reduced normal human gingival epithelial cell growth via an apoptosis/necrosis pathway involving Bax and Bcl-xL proteins and caspase-3 activity. Whole cigarette smoke also hindered epithelial cell migration, which in turn inhibited wound healing. This negative impact of smoke on gingival epithelial cells was confirmed by a disorganization of the engineered human oral mucosa when exposed to cigarette smoke. Further studies related to the mechanism of action of whole cigarette smoke are thus necessary to shed light on the connection between cigarette smoke and oral diseases such as cancer and periodontitis.

Acknowledgements

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